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# **GABA<sub>B</sub> receptor cell surface export is controlled by an endoplasmic reticulum gatekeeper**

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## Summary

Endoplasmic reticulum (ER) release and cell surface export of many G protein-coupled receptors (GPCRs), are tightly regulated. For GABA<sub>B</sub> receptors of GABA, the major mammalian inhibitory neurotransmitter, the ligand-binding GB1 subunit is maintained in the ER by unknown mechanisms in the absence of hetero-dimerization with the GB2 subunit. We report that GB1 retention is regulated by a specific gatekeeper, PRAF2. This ER resident transmembrane protein binds to GB1, preventing its progression in the biosynthetic pathway. GB1 release occurs upon competitive displacement from PRAF2 by GB2. PRAF2 concentration, relative to that of GB1 and GB2, tightly controls cell surface receptor density and controls GABA<sub>B</sub> function in neurons. Experimental perturbation of PRAF2 levels *in vivo* caused marked hyperactivity disorders in mice. These data reveal an unanticipated major impact of specific ER gate-keepers on GPCR function and identify PRAF2 as a new molecular target with therapeutic potential for psychiatric and neurological diseases involving GABA<sub>B</sub> function.

## Introduction

Cellular responses to hormones and neurotransmitters depend on the cell surface density of cognate receptors. Surface receptor number can vary considerably during adaptive responses to specific physiological constraints and in pathological conditions, both because of altered endocytosis <sup>1</sup> and changes in trafficking of receptors to the plasma membrane <sup>2</sup>. These processes have been intensively investigated for heptahelical receptors (also known as G protein-coupled receptors or GPCRs), which represent the largest family of plasma membrane receptors. For several GPCRs, the largest proportion of receptor molecules is not at the plasma membrane of native cells, but instead within internal stores <sup>3-7</sup>. Receptors can be mobilized from these stores upon external stimuli <sup>4, 8, 9</sup> targeting key components of the secretory pathway <sup>10</sup>, or after binding to endogenous ligands, which exert a chaperoning effect <sup>11</sup>. The cell surface delivery of these GPCRs is facilitated by a complex network of interactions with specific cellular chaperones or escort proteins <sup>5-7, 12-15</sup>, which allow the egress of receptors from the endoplasmic reticulum (ER) or release from the Golgi apparatus. Despite the identification of sequence motifs on cargo receptors, which control capture or release from intracellular stores, the molecular mechanisms of receptor retention are poorly understood. COP1 coatomer-dependent retrieval from the cis-Golgi to the ER was proposed in some cases <sup>16, 17</sup>, whereas retention via specific interaction with a ER resident gate-keeper was only reported during early development for Frizzled, a GPCR involved in the Wnt signaling pathway <sup>18</sup>.

Gamma-aminobutyric acid (GABA) is the principal inhibitory neurotransmitter in the vertebrate central nervous system. Its metabotropic GABA<sub>B</sub> receptor is a prototypical paradigm of an intracellularly-retained GPCR with regulated cell-surface

export <sup>19</sup>. Functional GABA<sub>B</sub> receptors are hetero-dimers constituted of two receptor protomers, referred to as GB1 and GB2 <sup>20-22</sup>. Two GB1 isoforms (GB1a and GB1b), produced as a consequence of a differential promoter usage in the *GB1* gene <sup>23</sup>, differ in their ectodomains by a pair of sushi repeats, which localize GABA<sub>B</sub> receptors to distinct synaptic sites, are. Although containing the GABA binding site in the extracellular domain <sup>20</sup>, GB1 isoforms fail to reach the cell surface when expressed in heterologous systems or overexpressed in neurons <sup>24</sup>. Indeed, GB1 contains an arginine-based signal in its carboxy-terminal tail, which causes its retention in the ER <sup>25, 26</sup>, and a di-leucine motif, which controls GB1 interaction with a guanine-nucleotide exchange factor and its exit from the trans-Golgi network <sup>27</sup>. GB2, on the other hand, does not bind to any known GABA<sub>B</sub> ligand <sup>28</sup> but is responsible for G-protein coupling <sup>29, 30</sup>. GB2 does not contain any retention signal and can reach the cell surface in the absence of GB1, as a functionally inactive homo-dimer <sup>31</sup>. It has been proposed that the shielding of the GB1 retention signal via a coiled-coil interaction with the carboxy-terminal of GB2 allows the hetero-dimer to reach the cell surface <sup>25</sup>, but the mechanistic aspects underlying GB1 retention have remained elusive. Although several proteins have been reported to interact with the coiled-coil domain of GB1 and to somehow regulate GABA<sub>B</sub>, none of them plays a direct role in GB1 retention in the ER: 14-3-3 $\zeta$  disrupts GB1-GB2 heterodimers at the cell surface <sup>32</sup>; upregulation of the transcription factor CHOP in response to ER stress interferes with GB1-GB2 heterodimerization <sup>33</sup>; the ARF1, 3,6 guanine-nucleotide exchange factor msec-7 controls GABA<sub>B</sub> exit from the Golgi apparatus <sup>27</sup>; the scaffolding protein GISP enhances GABA<sub>B</sub> receptor function by slowing receptor desensitization <sup>34</sup>.

Here we identified PRAF2, a 178 amino acid ER-resident protein <sup>35</sup> comprising 4 transmembrane domains with cytosolic amino- and carboxy-termini, as an essential

gatekeeper regulating GB1 release from the ER. PRAF2 is a member of the prenylated rab acceptor family, which includes the two structurally related proteins PRAF1 and PRAF3<sup>36</sup>. Golgi-resident PRAF1, was proposed to activate the dissociation of prenylated Rab proteins from the GDP dissociation inhibitor GDI, thus facilitating the association of prenylated Rabs with target Golgi membranes<sup>37</sup>. PRAF3, the mammalian analogue of the yeast protein Yip6b, was reported to delay ER exit of the Na<sup>+</sup>-dependent glutamate transporter Excitatory Amino-Acid Carrier 1 (EAAC1)<sup>38, 39</sup>. PRAF2 identified in a yeast two-hybrid screen as an interacting partner of the chemokine receptor CCR5<sup>40</sup>, was found enriched in human brain<sup>41</sup> and overexpressed in multiple cancers<sup>36, 42</sup>. However, no specific biological function has so far been attributed to PRAF2.

Our data demonstrate that PRAF2 interaction with GB1 controls GABA<sub>B</sub> function at the cell surface of neurons and *in vivo*.

## Materials and Methods

Extensive description of materials and methods can be found in [Supplementary Material](#).

## Results

### *PRAF2 interacts with GB1 in the ER of transfected fibroblasts and primary neurons*

In the search for a potential gatekeeper retaining GB1 in the ER until it associates with GB2, we investigated whether the ER-resident PRAFs, PRAF2 and PRAF3 might be interacting partners of GB1. Co-immunoprecipitation experiments were conducted in HEK-293 cells expressing tagged proteins. GB1-GFP (the GB1b isoform were used in transfected fibroblasts throughout this study) was found to co-immunoprecipitate (co-IP) with V5-PRAF2, whereas almost no co-IP was observed with V5-PRAF3 ([Figure 1a](#)). V5-PRAF2 interacted with GB1 but not GB2 ([Supplementary Figure S1a](#)). Based on these observations, subsequent studies focused on PRAF2. To confirm that the interaction between the receptor and PRAF2 occurs in the ER, the glycosylation profile of GB1 that co-immunoprecipitated with PRAF2 was examined ([Supplementary Figure S1b](#)). N-glycans added in the ER are sensitive to both Endo H and PNGase F, whereas subsequent glycosylation in the Golgi is resistant to Endo H and sensitive to PNGase F <sup>43</sup>. Following co-IP with PRAF2, GB1 was deglycosylated by both enzymes in the presence or absence of co-expressed GB2. In the presence of GB2 most GB1 underwent N-glycosylation in the Golgi, protecting the receptor against Endo H action in the cell lysate ([Supplementary Figure S1b](#), upper panel). These data therefore demonstrate that the GB1, which co-IP with PRAF2 is from the ER and not from post ER compartments.

In agreement with previous studies <sup>36</sup>, PRAF2 was found to be present in many cell lines and abundant in the brain (Figure 1b). Likely due to its highly hydrophobic domains, endogenous PRAF2 may form oligomers, which are visible after electrophoretic separation at low stringency <sup>40</sup>. Accordingly, we detected variable combinations of monomeric ( $\approx 20$ kDa) dimeric ( $\approx 40$ kDa) and high molecular weight forms, in rat and mouse tissue or cell extracts and in human cell extracts. In all cases, immunostained material did correspond to PRAF2, as confirmed by the loss of signal following RNA interference (Supplementary Figure S2a).

Endogenous GB1a/GB1b (Figure 1b) and PRAF2 from adult rat brain extracts co-immunoprecipitated (Figure 1c), indicating that both receptor isoforms can be found in a molecular complex with PRAF2. The subcellular distribution of these proteins was studied in embryonic (E17) rat hippocampal neurons grown in culture. Confocal immunofluorescence analysis on permeabilized cells showed marked cytoplasmic co-localization of endogenous GB1 and PRAF2 ( $80.2 \pm 5.6\%$ , Figure 1d). PRAF2 staining was particularly abundant in the cell body, but also present in neurites. GB1 staining (Supplementary Figure S3a for GB1 antibody specificity) overlapped with both ER (KDEL receptor) and Golgi (GM130) markers, whereas PRAF2 was mainly colocalized with the ER marker ( $76.7 \pm 6.7\%$ , Figure 1d), consistent with previous studies <sup>42</sup>.

#### *PRAF2 controls GB1 export and regulates GABA<sub>B</sub> function at the cell surface of neurons*

Based on the experiments above, GB1 and PRAF2 likely interact in the ER, where GB1 is retained in the absence of hetero-dimerization with GB2. To investigate whether PRAF2 could control the exit of GB1 from the ER, hippocampal neurons



were transfected with siRNAs to down-modulate PRAF2 expression. One week after transfection with specific siRNAs, neurons expressed 10-30% residual PRAF2 (Figure 2a and Supplementary Figure S2a). To measure the amount of surface binding sites, binding studies on intact cells were conducted on the same samples using [<sup>3</sup>H]-CGP54626, a hydrophilic membrane impermeable GABA<sub>B</sub> antagonist, which specifically binds to the GB1 isoform and not to GB2<sup>28</sup> (see also Supplementary Figure S3c). An up to three-fold increase of surface GB1 was measured in cells with decreased PRAF2 expression compared to controls (Figure 2a), consistent with the hypothesis that PRAF2 may function as a GB1 gatekeeper in the ER. In line with this, expression of exogenous PRAF2 in hippocampal neurons following infection with adenoviruses carrying a PRAF2 construct (AAV-PRAF2+GFP) was associated with a 60% decrease of surface GB1 binding sites (Figure 2b). In contrast, and in agreement with their lack of interaction in co-IP experiments, exogenous PRAF2 did not modify the amount of cell surface GB2 in AAV-PRAF2+GFP-infected cells (Supplementary Figure S1c and Supplementary Figure S3b for GB2 antibody specificity).

We next examined whether the additional surface GB1 detected upon PRAF2 inhibition was associated with GB2 within a GABA<sub>B</sub> hetero-dimer or released alone. Since GABA<sub>B</sub> coupling to G proteins exclusively depends on the GB2 subunit, agonist-promoted functional outputs depend on the formation of GABA<sub>B</sub> hetero-dimers<sup>44</sup>. Patch-clamp experiments were therefore conducted to measure the inhibitory effect of the GABA<sub>B</sub> agonist baclofen on the spontaneous electric activity of hippocampal neurons in culture (Figure 2c-f, Supplementary Figure S2 and Table S1). The basal spontaneous electrical activity was comparable in untreated neurons, neurons treated with the various siRNAs or infected with AAV-PRAF2+GFP (Figure

2c). Baclofen caused a dose-dependent inhibition of the spontaneous electrical activity of neurons (Figure 2e), the effect being reversed by pre-incubation with the GABA<sub>B</sub> antagonist CGP54626 (Figure 2d). Both PRAF2-specific siRNAs led to a leftward shift of the inhibition curve compared to untreated cells or cells receiving scrambled siRNA (Figure 2e), indicating that the inhibition of the spontaneous activity in PRAF2-depleted neurons was much more efficient at lower concentrations of baclofen, as a consequence of an increased number of surface GABA<sub>B</sub> hetero-dimers. Conversely, virus-induced overexpression of PRAF2 impaired the effect of baclofen, indicating a decreased number of surface GABA<sub>B</sub> hetero-dimers (Figure 2f). Thus, the PRAF2-dependent regulation of GB1 cell surface delivery controls the density of functional GABA<sub>B</sub> hetero-dimers, directly impacting on the inhibitory threshold of GABA signaling.

#### *Competition between GB2 and PRAF2 determines the release of GB1 from the ER*

The molecular mechanism of GB1 regulation by PRAF2 was investigated in a reconstituted cell model. The cell surface export of GB1 was measured by FACS analysis in transfected HEK-293 cells (see [Supplementary Experimental Procedures](#)). In the absence of co-expressed GB2, minor or no amounts of exogenous GB1 are detectable at the surface of transfected cells, depending on the cell type and on the experimental method<sup>25, 26</sup>. Accordingly, in the absence of GB2, extracellular GB1 only represented 5 to 10% of the maximal signal measured upon GB1 and GB2 co-expression in transfected HEK-293 cells (Figure 3a). By increasing the amounts of exogenous PRAF2 in cells expressing constant amounts of GB1 and GB2, a progressive decrease of surface GB1 was observed (Figure 3a). These data suggest that the actual amount of GB1 reaching the cell surface is controlled by GB2

and PRAF2, these proteins exerting opposite effects on forward trafficking. In contrast, PRAF2 was unable to modulate the cell surface targeting of endogenous GB2 in neurons ([Supplementary Figure S1c](#)) or exogenous GB2 expressed alone in HEK-293 cells ([Supplementary Figure S1d](#)), consistent with the observation that GB2 does not interact with PRAF2 ([Supplementary Figure S1a](#)).

Immunofluorescence experiments were then conducted to examine how the subcellular distribution of GB1 is modulated in HEK-293 cells by the co-expression of GB2 and PRAF2 ([Supplementary Figure S5](#)). When expressed alone, GB1-GFP was largely intracellular, co-localizing with the ER marker BIP ( $92.9 \pm 1.3\%$ , [panels a,f](#)). In the presence of co-expressed GB2-HA, a substantial proportion of GB1-GFP was translocated to the plasma membrane area where it co-localized with GB2-HA ( $95.4 \pm 0.9\%$ , [panels b,c,g](#)); if PRAF2-V5 was overexpressed together with GB2-HA, GB1-GFP mostly recovered its original intracellular distribution ( $80.8 \pm 2.6\%$  colocalization with BIP, [panels d,h](#)) and appeared less localized with GB2-HA ( $53.4 \pm 3.2\%$ , [panel e](#)). Most co-localized PRAF2-V5/GB1-GFP signal also co-localized with the anti-BIP signal ( $98.1 \pm 0.5\%$ , [panel d](#)).

These data indicate that the relative stoichiometry of GB1, GB2 and PRAF2 determines the actual amount of surface GABA<sub>B</sub> heterodimer, GB2 and PRAF2 competing for GB1 association. The capacity of GB2 to displace the GB1-PRAF2 interaction was assessed by co-IP experiments in the presence of increasing concentrations of GB2: for a constant concentration of PRAF2, increasing GB2 expression progressively reduced the amount of GB1 recovered in complex with PRAF2 ([Figure 3b](#)). Bioluminescence Resonance Energy Transfer (BRET) experiments, permit monitoring of the proximity of protein partners in intact cells<sup>45, 46</sup>. PRAF 2 cDNA was fused with the coding region of Renilla luciferase (Rluc), the

BRET donor, and the GB1 coding region was fused upstream to that of the yellow variant (YFP) of the green fluorescent protein (GFP), the BRET acceptor. Saturation BRET experiments <sup>46</sup> were then conducted by transfecting constant amounts of donor plasmid and increasing amounts of acceptor plasmid. Hyperbolic curves indicative of specific GB1-PRAF2 proximity (Figure 3c) were obtained in the absence or presence of increasing amounts of HA-tagged GB2. However, BRET<sub>50</sub> values, which reflect the propensity of the investigated proteins to be in close proximity, were increased in the presence of HA-GB2, consistent with a model of competitive inhibition of GB1-PRAF2 interaction by GB2. Accordingly, the capacity of GB2 to displace the GB1-PRAF2 interaction was correlated with increased expression of GB1 at the cell surface (Figure 3d).

The RSR motif present in the C-terminus of GB1 participates in its ER retention <sup>25</sup>, as shown by the enhanced cell surface expression of the GB1-ASA mutant (alanine substitution of the arginine residues of the RSR motif) even in the absence of GB2 <sup>25-27</sup>. We hypothesized that this RSR motif could participate in the interaction of GB1 with PRAF2. The amount of GB1-ASA co-immunoprecipitated with PRAF2 was decreased compared to wt GB1 (Figure 3e), confirming the above hypothesis, but also indicating that the interface between these proteins may involve other motifs. A di-leucine motif upstream of the RXR motif also participates in the control of cell surface export of GB1, the GB1-AA-ASA mutant (alanine substitution of both RXR and di-leucine motifs), being better exported to the cell surface than wt GB1 or the single GB1-ASA mutant in the absence of GB2 <sup>25</sup>. We compared the interaction of GB1, GB1-ASA GB1-AA and GB1-AA-ASA with PRAF2 in co-IP experiments (Figure 3e) and found that the di-leucine motif participates in the association with PRAF2. The substitution of both LL and RXR motifs abolished the

interaction of GB1 and PRAF2. It was proposed that the LL motif might participate in the interaction of GB1 with a guanine-nucleotide exchange factor in the trans-Golgi network <sup>27</sup>. Our data indicate that the di-leucine motif may also contribute to GB1 retention in the ER.

*Increased expression of PRAF2 in ventral tegmental neurons in mice causes major hyperactivity*

We next investigated whether perturbing the stoichiometry of GB1, GB2 and PRAF2 might affect GABA<sub>B</sub>-dependent regulations *in vivo*. GABA<sub>B</sub> receptor function was studied in the ventral tegmental area (VTA), a brain area containing GABA<sub>B</sub>-expressing neurons <sup>47, 48</sup>. Activation of the dopaminergic circuits from the VTA to the nucleus accumbens produces hyperlocomotor activity <sup>49</sup> and GABA<sub>B</sub>-mediated activation of slow inhibitory currents plays an important role in the control of dopaminergic neuron excitability in the VTA <sup>48, 50</sup>. Somatodendritic GABA<sub>B</sub> receptors inhibit dopamine release by causing K<sup>+</sup>-dependent hyperpolarization, which decreases burst firing-activity of dopaminergic neurons <sup>48, 51</sup>, whereas presynaptic GABA<sub>B</sub> receptors curtail dopamine release by inhibiting Ca<sup>2+</sup> influx <sup>52</sup>. We predicted that the loss of surface GABA<sub>B</sub> receptors in VTA dopaminergic neurons resulting from a local rise in PRAF2 expression and GB1 retention, would contribute to increased dopamine release and subsequent enhanced locomotor activity. Bilateral stereotaxic injection of the bicistronic adenoviral PRAF2 and GFP delivery system (AAV-PRAF2+GFP) into the VTA led to a dramatic dose-dependent increase in basal locomotor activity, compared to animals receiving a virus that only expressed GFP (AAV-GFP) ([Figure 4a](#)). Mouse brains were examined to verify the distribution of adenovirus-encoded exogenous PRAF2 and GFP. GFP expression was mostly

restricted to the VTA and occurred in both tyrosine-hydroxylase-positive (dopaminergic) and negative neurons (Supplementary Figure S6a). Habituation to the novel environment had no effect in AAV-PRAF2+GFP-injected mice, whereas AAV-GFP-injected animals showed a progressive reduction in locomotor activity during the session (over the 60min period) and compared to the first day (Figure 4a,b).

To establish the amount of exogenous PRAF2 responsible for these motor phenotypes, PRAF2 was quantified in the VTA by immunoblot experiments with anti-PRAF2 antibodies (Supplementary Figure S6b). A linear correlation was observed between PRAF2 concentration and motor activity, the latter being enhanced even for less than a two-fold increase in PRAF2 concentration. Interestingly, variations in PRAF2 concentration of a comparable or even larger extent are observed in different areas of the brain and different cell types (Figure 1b) and during hippocampal neuron maturation in culture (Supplementary Figure S6c). Together, these data indicate that moderate changes of PRAF2 expression *in vivo* cause significant phenotypic effects.

#### *Correlation between PRAF2-induced hyperactivity and GABA<sub>B</sub> function in the VTA*

Although consistent with impaired availability of GABA<sub>B</sub> sites at the cell surface of VTA neurons, the phenotypic effect of AAV-PRAF2+GFP injection might also be due to the involvement of other receptors or transporters similarly retained by PRAF2 in the ER. To establish a correlation between the observed phenotype and GABA<sub>B</sub> function in the VTA, we used several approaches.

Single-cell electrophysiological experiments were performed in dopaminergic neurons of the VTA expressing exogenous PRAF2, to analyse local response to the GABA<sub>B</sub> agonist baclofen. AAV-PRAF2+GFP-infected dopaminergic VTA neurons

displayed a markedly impaired current response to baclofen compared to control AAV-GFP-containing neurons, fully consistent with reduced expression of GABA<sub>B</sub> at the cell surface (Figures 4c,d,e).

Baclofen stimulation of GABA<sub>B</sub> receptors *in vivo* inhibits the enhanced locomotor activity arising from acute stimulation of VTA neurons by amphetamine<sup>53-55</sup>. Consistently, baclofen pretreatment completely inhibited the acute effect of amphetamine in mice bilaterally injected in the VTA with the control virus (Figure 5a). In contrast, although amphetamine administration further enhanced locomotor activity in mice injected with the AAV-PRAF2+GFP virus, baclofen pretreatment failed to abolish this effect, supporting the impairment of GABA<sub>B</sub> receptor inhibitory activity in this context (Figure 5b). Interestingly, the VTA-selective bilateral ablation of GB1, achieved by injecting a virus carrying the CRE recombinase (AAV-CRE) in both VTAs of GB1-foxed mice (GB1<sup>fl/fl</sup>), pheno-copied the behavioral effect induced by AAV-PRAF2+GFP in WT mice (Figures 5c and Supplementary Figure S7a). The motor activity of these mice was enhanced by acute administration of amphetamine, but baclofen failed to inhibit the motor effect of amphetamine, similarly to what was observed in wt mice injected with AAV-PRAF2+GFP (compare Figures 5b and 5c).

We next examined the effect of unilateral injection of AAV-PRAF2+GFP virus into the VTA of normal animals or mice displaying global GB1 knock-out<sup>56</sup>. GB1-KO mice exhibit spontaneous hyperlocomotor activity (Figure 5d,e pre-test). Previous studies reported that asymmetric alteration of basal ganglia activity contributes to a rotational behavior<sup>57</sup>; the unilateral increase of dopaminergic activity in the mesolimbic circuit causes contralateral pivoting<sup>58, 59</sup>, whereas ipsilateral pivoting is usually associated with reduced activity in the same path<sup>60</sup>. After unilateral injection of AAV-PRAF2+GFP virus into the VTA, leading to a two-fold local increase of

PRAF2 (Supplementary Figure S7b,c), normal control littermates of the GB1-KO mice showed both a significant increase of their overall locomotor activity (Figure 5d,e) and a contralateral turning phenotype (Figure 5f, Movie S1a) compared to control AAV-GFP-injected mice (Movie S1b), confirming that dopaminergic neurons were over-activated in the injected VTA. In contrast, consistent with a functional link between the effect of PRAF2 overexpression and impaired GB1 expression, the unilateral AAV-PRAF2+GFP injection in the VTA of GB1-KO mice neither increased overall activity (Figure 5d,e) nor affected the spontaneous turning phenotype, which was equally contralateral or ipsilateral (Figure 5f). Interestingly, the unilateral deletion of GB1 in the VTA of GB1<sup>fl/fl</sup> mice (Supplementary Figure S7a) recapitulated the hyperlocomotor activity and the contralateral turning phenotype observed in wt mice receiving unilateral injection of AAV-PRAF2+GFP in the VTA (Figure 5d-f). Together, these data fully support the hypothesis that the behavioral changes induced by modulating the content of PRAF2 in the VTA depend on local GABA<sub>B</sub> function.

In conclusion, moderate changes of PRAF2 concentration are sufficient to markedly affect cell surface GB1 targeting and, consequently, GABA<sub>B</sub> function both *in vitro* and *in vivo*, via GB1 retention in the ER.



## Discussion

PRAF2 functions as an ER gatekeeper that prevents GB1 from egressing the ER until it is competitively displaced by GB2. Thereafter, the GB1-GB2 hetero-dimer can progress to the Golgi apparatus and subsequently to the plasma membrane as a functional GABA<sub>B</sub> receptor.

The identification of PRAF2 as a reticular GB1 tether uncovers a central piece of a puzzle initiated by the discovery that GABA<sub>B</sub> receptors are hetero-dimers of GB1 and GB2 subunits and that the RXR signal, contained in the carboxy-terminal tail of GB1, participates in GB1 ER retention. A simple mechanism of control of receptor function also emerges from our data, which does not rely on gene transcription or enzymatic protein modification, but rather on the competition between a gatekeeper (PRAF2) and GB2 for interaction with GB1. The fact that cell surface GB1 is dependent on the relative concentrations of competing proteins with opposite effects on its forward trafficking might also explain how functional (although leading to atypical outputs) cell surface GB1 is found in some brain areas of GB2-KO mice<sup>61</sup>. PRAF2 might be less abundant in these particular areas and thus insufficient for retaining all GB1 molecules, which could then reach the cell surface as monomers or homo-dimers. Alternatively, in the absence of GB2, GB1 could hetero-dimerize with another ER-resident GPCR and escape PRAF2 retention.

The concentration of PRAF2 in brain areas is likely submitted to regulation, as, for example, PRAF2 concentration can vary up to 20-fold in embryonic hippocampal neurons after a few days in culture. On the other hand, less than twofold changes in the VTA are sufficient to promote major motor hyperactivity. The mechanistic role of impaired GB1 expression at the surface of VTA neurons in our hyperactive mice was substantiated by several experimental lines of evidence: i) the loss of baclofen-

mediated inhibition of the motor effects caused by amphetamine both in wt mice expressing exogenous PRAF2 in the VTA and mice with localized deletion of GB1 in the VTA; ii) the absence of specific PRAF2 effect in GB1-KO mice; iii) the impaired functional response to baclofen in VTA neurons expressing exogenous PRAF2. It is also consistent with the observation that GB1- or GB2-KO mice exhibit enhanced hyperlocomotor activity <sup>56, 61-63</sup>, and that GABA<sub>B</sub> antagonists stimulate <sup>64</sup>, whereas intra-VTA agonists depress basal locomotor activity <sup>65</sup>.

PRAF2 belongs to the short list of specific ER-resident proteins that inhibit the forward export of plasma membrane proteins. These include, Rer1p, which negatively regulates gamma-secretase complex assembly <sup>66</sup>, and Shisa a GPCR-interacting, ER-resident protein controlling the surface density of Frizzled during development <sup>18</sup>. Among the other PRAFs, PRAF3, which was reported to somehow control the cell surface function of the Excitatory Amino-Acid Carrier 1 <sup>38, 39</sup>, might similarly function as an ER gatekeeper for this protein. So far, although not excluded, there is no experimental evidence that PRAF1 might be a Golgi gatekeeper.

Whereas GABA<sub>B</sub> is the only GPCR so far with an identified ER gatekeeper, experimental data suggest that additional members of this receptor family might be controlled by an analogous mechanism. Indeed, either the existence of abundant stores in the ER of primary cells, or regulated delivery to the cell surface were reported for several other GPCRs <sup>6</sup>. In most cases, their association with private chaperones or with escort proteins promoted exit from the ER <sup>7, 12, 67, 68</sup>. In the context of GPCR export in neurons, a depression-like phenotype was observed in knockout mice for p11 <sup>14</sup> a member of the S100 EF-hand calcium-dependent signaling modulators <sup>69</sup>, which specifically interacts with serotonin 5-HT<sub>1B</sub> receptors

(5-HT<sub>1B</sub>Rs). Cell surface density and function of 5-HT<sub>1B</sub>Rs were decreased in these mice, indicating that p11 is an escort for 5-HT<sub>1B</sub>Rs. Interestingly, adenovirus-mediated transfer of the gene encoding p11 to the nucleus accumbens rescued the depression-related behavioral disorders of p11 knockout mice<sup>70</sup>. In light of the data reported here, it is plausible that gatekeepers regulate at least some of these GPCRs in a similar fashion to GB1.

Clinical implications of impaired GPCR export from internal compartments to the cell surface have been documented for several diseases and receptors<sup>71, 72</sup>. In most cases the mechanism of retention was attributed to receptor mutations. Based on our study in mice, a pathological deregulation of gatekeeper content could represent an additional mechanism in this context. Consistent with this hypothesis, a two-threefold increase of *PRAF2* mRNA was reported in human brain tumors compared to normal samples<sup>36, 42</sup>.

In conclusion, we have uncovered a mechanism by which GPCR function can be regulated by the competing effects exerted by gatekeepers and escorts on receptor trafficking to the cell surface. Although interfering with this process causes major pathological effects in mice models, further studies are necessary to appreciate whether deregulation of gatekeeper and/or escort expression levels are pathogenic in human diseases.

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## Figure legends

### Figure 1. Interaction of PRAF2 and GB1.

(a) HEK-293 cells were transfected with plasmids coding for GB1-GFP and/or V5-epitope tagged PRAF2 or PRAF3, as indicated. Immunoprecipitation with a monoclonal anti-V5 antibody was performed from 1mg protein of cell lysates. The presence of GB1 and PRAFs was revealed with anti-GFP and anti-V5 antibodies, respectively. 50 µg of the inputs were analyzed to determine GB1-GFP expression. Lower panel: densitometric analysis of GFP immunoreactivity, corresponding to the GB1-GFP that was co- immunoprecipitated with PRAF-V5 upon incubation with the V5 antibody; AU: arbitrary units; Unpaired t-test \*\*\*\*  $p < 0.0001$  (N=3). (b) PRAF2 and GB1 expression in human (Hela, LN229, U87, U373, THP-1) and mouse cell lines, rat brain, neurons and astrocytes. Immunoblot experiments with Anti-PRAF2 and Anti-GB1 antibodies (see [Supplementary Figure S3a](#) for monoclonal anti-GB1 antibody specificity) were conducted on 50µg of total proteins after cell lysis. M: monomeric PRAF2; D: dimeric PRAF2; H: high molecular weight forms likely corresponding to PRAF2 multimers. PRAF2 is also expressed in HEK-293 cells (not shown). (c) Coimmunoprecipitation study of endogenous GB1 and PRAF2 from adult rat brains. Experiments were performed on 12mg solubilized brain proteins obtained as described in methods. Samples were immunoprecipitated with control IgG or anti-PRAF2 antibodies, separated by SDS-PAGE and immunoblotted with anti-GB1 or anti-PRAF2 antibodies. 50µg input was co-migrated for comparison. (d) Subcellular distribution of PRAF2 and GB1 in rat hippocampal neurons in culture. Permeabilized cells were stained with anti-PRAF2 and anti-GB1 antibodies and antibodies directed against KDEL, to label the ER, or the Golgi marker GM130, then incubated with the

appropriate secondary antibodies coupled to green or red fluochromes. Co-localization (orange in merge panels) was quantified using Image J software. PRAF2 mostly overlapped with KDEL staining ( $76.7\% \pm 6.7\%$ ) and a minor fraction overlapped with GM130 staining ( $8.88 \pm 1.1\%$ ) in 3 independent experiments; PRAF2 and GB1 overlap was  $80.2 \pm 5.6\%$ ; GB1 and KDEL overlap was  $79.7 \pm 2.5\%$ . Scale bar: 10  $\mu\text{m}$ . Insets: enlarged areas.

**Figure 2. PRAF2 modulation affects cell surface GB1 expression and function in neurons.**

(a) Hippocampal neurons in culture for 7 days were transfected with control-scrambled or PRAF2-specific siRNAs (Si1 and Si2). After one week, the efficacy of PRAF2 gene silencing was determined by immunoblot experiments on 50 $\mu\text{g}$  of total cell lysates using anti-PRAF2 antibodies ([see Supplementary Figure S2a](#)). Residual PRAF2 after specific siRNA treatment was compared to values in neurons treated with control siRNA (yellow boxes, n=12). Determination of GB1 binding sites at the surface of siRNA-treated neurons was performed by a radioligand assay, using the hydrophilic GB1-specific ([see Supplementary Figure S3c](#)) antagonist [ $^3\text{H}$ ]CGP54626 as ligand. One week after transfection, neurons ( $60\text{--}90 \times 10^3$  per well) were incubated in culture wells with 20 nM [ $^3\text{H}$ ]CGP54626 for 20 min at RT, in the absence or presence (to determine non-specific binding) of 10  $\mu\text{M}$  unlabeled CGP54626 (see methods). The number of binding sites after specific siRNA treatment was compared to values in neurons treated with control siRNA (red boxes, n=12). One-way ANOVA  $^{**}p < 0.02$ ;  $^{***}p < 0.001$ . (b) Expression of exogenous PRAF2 in neurons inhibits the cell-surface density of GABA<sub>B</sub> binding sites. Neurons infected with an adenovirus allowing the simultaneous expression of PRAF2 and GFP (AAV-PRAF2+GFP). Upper part: transmission and immunofluorescence microscopy images showing

infected neurons in green; lower part: ligand-binding assay in the same neurons performed as in panel (a); Unpaired t-test  $n=3$   $*p<0,05$ . (c) Basal spontaneous activity recorded in hippocampal neurons infected with AAVs (as indicated) or transfected with siRNAs, before baclofen treatment. (d) The inhibition of spontaneous activity caused by  $0.2\mu\text{M}$  baclofen was measured in additional untreated neurons in the absence or presence of the GABA<sub>B</sub> antagonist CGP54626 ( $100\text{ nM}$ ),  $**p<0.01$ . (e) Dose-response curves of baclofen-induced inhibition of spontaneous action potential discharge were established in untreated neurons, neurons transfected with control siRNA or with PRAF2-specific siRNAs 1 or 2. Data were normalized to neurons under basal conditions and analyzed using GraphPad Prism software, using the one-site inhibition curve as model. The number of recorded neurons for each condition is indicated. Calculated  $\text{IC}_{50}$  values were: untreated,  $7.2 \times 10^{-7}\text{M}$  (95%CI:  $3.0 \times 10^{-7}$ - $1.71 \times 10^{-6}$ ); Si-control,  $4.5 \times 10^{-7}\text{M}$  (95%CI:  $1.8 \times 10^{-7}$ - $1.11 \times 10^{-6}$ ); Si1-PRAF2,  $8.0 \times 10^{-8}\text{M}$  (95%CI:  $4.8 \times 10^{-8}$ - $1.41 \times 10^{-7}$ ); Si2-PRAF2,  $1.3 \times 10^{-7}\text{M}$  (95%CI:  $8.3 \times 10^{-8}$ - $2.21 \times 10^{-7}$ ). Differences between the various conditions were also analyzed for each concentration of baclofen in the [Table S1](#). (f) Comparison of the inhibitory effect of  $0.2\mu\text{M}$  baclofen in untreated neurons, Si-control or Si(1+2)-PRAF2-treated neurons, and neurons infected with AAV-PRAF2+GFP. (\*\*\*\*  $p<0.0001$ , untreated versus Si(1+2)-PRAF2;  $*p<0.05$ , untreated versus AAV-PRAF2+GFP; Student T-test). For sample current recordings see [Supplementary Figure S2b](#).

### **Figure 3. Surface GB1 is regulated by PRAF2 and GB2 expression level.**

(a) HEK-293 cells were transiently cotransfected with the indicated constant amount of myc-GB1-YFP and HA-GB2 plasmid DNA and increasing concentrations of



PRAF2 construct. Surface GB1 in YFP-positive, non-permeabilized cells was quantified by FACS analysis using the 9E10 anti-Myc monoclonal primary antibody and a secondary anti-mouse Cy5-conjugated antibody ([Supplementary Figure S4](#)). Cell surface GB1 expression is shown by the histograms: bars indicate SEM from three experiments in duplicate. The amount of the indicated proteins is shown by the immunoblots below the histograms. **(b-d)** GB2 competes with PRAF2 for GB1 interaction. **(b)** Competition between PRAF2 and GB2 for interaction with GB1 in co-IP experiments. HEK-293 cells were transfected with the indicated plasmids. Immunoprecipitation with a monoclonal anti-V5 antibody was performed from 1mg protein of cell lysates. The presence of GB1, GB2 and PRAFs was revealed with anti-GFP, anti-HA and anti-V5 antibodies, respectively. 50  $\mu$ g of the inputs were analyzed by immunoblot to determine the expression level of the proteins. Lower histograms: densitometric analysis of GFP immunoreactivity, corresponding to the GB1-GFP that was co-immunoprecipitated with PRAF-V5 in the presence of increasing amounts of GB2. \* $p < 0.01$  \*\*\* $p < 0.001$ , one-way ANOVA ( $n = 3$ ). **(c)** BRET saturation experiments of PRAF2-Rluc donor with GB1-YFP acceptor were conducted in the absence or presence of two different amounts of unlabeled GB2 (50ng or 250ng plasmid DNA). Calculated BRET<sub>50</sub> values were:  $1.4 \pm 0.3$  (GB2=0)  $4.9 \pm 1.1$  (GB2=50) and  $12.4 \pm 2.5$  (GB2=250);  $n = 22$  to 66 independent transfections per plot (significant difference between GB2=0 and GB2=250 ( $p < 0.01$ ), and between GB2=50 and GB2=250 ( $p < 0.05$ ), one-way ANOVA). **(d)** HEK-293 cells were co-transfected with the constant amounts of myc-GB1-YFP and PRAF2-V5 plasmids and increasing concentrations of HA-GB2 plasmid. Cell surface export of GB1 was examined by FACS on non-permeabilized cells, as described in panel a. **(e)** Involvement of the GB1 RXR and LL motifs in the interaction with PRAF2. Co-

immunoprecipitation experiments conducted as in [Figure 1a](#) comparing the association of GB1-GFP, GB1-ASA-GFP, GB1-AA-GFP and GB1-AA/ASA-GFP with PRAF2-V5; bottom: quantitation of immunoprecipitated material. Tub: tubulin loading control; \*\*\* $p < 0.0001$  \*\*\*\* $p < 0.00001$ , one way Anova, from 4 independent experiments.

**Figure 4. Locomotor activity and baclofen-evoked GABA<sub>B</sub> currents in mice expressing exogenous PRAF2 in the VTA**

(a) Locomotor activity and habituation to a novel environment of mice receiving bilateral injection in the VTA of AAV-GFP (open symbols) or AAV-PRAF2+GFP (filled symbols) after 1 (Day 1), 2 (Day 2) or 3 (Day 3) days of habituation. Locomotor activity was measured in a circular corridor with four infrared beams placed every 90°. Counts corresponding to consecutive interruption of two adjacent beams (i.e., mice moving through one-quarter of the corridor) were incremented every 5 min. The effects of 200 or 500 nL of injected virus are shown ( $n=16$  in each group). For open symbols, error bars are smaller than the size of the symbols. See [Supplementary Figures S6-7](#) for quantitative analysis of PRAF2 expression in the VTA. (b) Habituation had no effect in AAV-PRAF2+GFP-injected mice (lower panels), whereas AAV-GFP-injected mice (upper panels), showed reduced locomotor activity at days 2 and 3 (D2 and D3) compared to the first day (D1). Data were analyzed using two-way ANOVA (means $\pm$ SEM) \*\*\*\* $P < 0.0001$  Df(1). (c-e) Overexpression of PRAF2 in the VTA reduces baclofen-evoked GABA<sub>B</sub> currents. (c) Visualization of the injection area of AAV-PRAF2-GFP or control AAV-GFP (left panel) and of transfected neurons (middle panel). Three weeks after injection, GFP-positive VTA neurons were patch-clamped under visual guidance (right panel) to assess baclofen-induced currents.

Scale bar, 20  $\mu\text{m}$  (**d**). Left panel: example of traces of VTA GFP-positive neurons exhibiting *h*-current (left scale bars 200 ms and 100 pA) in response to bath-application of baclofen (15  $\mu\text{M}$ ) with an outward current, which was blocked by the GABA<sub>B</sub>R-antagonist CGP52432 (5  $\mu\text{M}$ ) (right scale bars 2min and 50 pA). (**e**) Peak maximal current in putative VTA dopamine neurons infected with either control AAV-GFP (black column), or AAV-PRAF2+GFP (red column). A significant decrease in the maximal baclofen-induced current was observed in neurons expressing exogenous PRAF2 (Control:  $220.65 \pm 57.24$  pA,  $n=8$ ; AAV-PRAF2+GFP:  $74.78 \pm 30.38$  pA,  $n=10$ ;  $t(16)=2.383$ ,  $*p<0.05$ ). The baclofen-induced current was typically partly desensitized, reaching a steady state current (*I* steady). Compared to control neurons, VTA dopamine neurons expressing exogenous PRAF2 displayed reduced steady state currents (Control:  $156.63 \pm 43.32$  pA,  $n=8$ ; AAV-PRA2+GFP:  $59.59 \pm 19.64$  pA,  $n=10$ ;  $t(16)=2.189$ ,  $p<0.05$ ), and also exhibited less desensitization (*I* max – *I* steady) currents (Control:  $64.03 \pm 19.38$  pA,  $n=8$ ; AAV-PRAF2+GFP:  $15.19 \pm 12.23$  pA,  $n=10$ ;  $t(16)=2.217$ ,  $p<0.05$ ).

**Figure 5. Behavioral changes induced by modulating the content of PRAF2 in the VTA depend on local GABA<sub>B</sub> function.**

(**a-c**) Overexpression of PRAF2 in the VTA reduces baclofen dependent behavioral effects. The effect of baclofen (4mg/kg, i.p.) on amphetamine-induced locomotor activity was examined in wt mice (**a**, **b**) injected bilaterally with 200nL of AAV-GFP (**a**) or AAV-PRAF2+GFP (**b**) ( $n=8$  in each group) or GB1<sup>fl/fl</sup> mice injected bilaterally with 200nL of AAV-CRE-GFP (**c**) ( $n=6$ ). Animals received saline (open symbols) or baclofen (filled symbols) 30min before amphetamine (3mg/kg, i.p. squares) or saline (circles) administration. Locomotor activity was then recorded for 2h. Data (means  $\pm$

SEM,  $n = 6-8$  per group) were analyzed by two-way ANOVA with treatment as main factor ( $F(5,31)=6,083$   $P=0,0005$ ), revealing the following significant interactions: AAV-PRAF2+GFP saline+AMPH vs. AAV-GFP baclo+AMPH ( $p<0.001$ ); AAV-PRAF2+GFP baclo+AMPH vs. AAV-GFP baclo+AMPH ( $p<0.001$ ); AAV-GFP saline+AMPH vs. AAV-GFP baclo+AMPH ( $p<0.01$ ). AAV-GFP baclo+AMPH vs. AAV-CRE saline+AMPH ( $P<0.001$ ); AAV-GFP baclo+AMPH vs. AAV-CRE baclo+AMPH ( $p<0.001$ ). No interaction was found for: AAV-PRAF2+GFP saline+AMPH vs. AAV-PRAF2+GFP baclo+AMPH; AAV-PRAF2+GFP saline+AMPH vs. AAV-GFP saline+AMPH; AAV-PRAF2+GFP baclo+AMPH vs. AAV-GFP saline+AMPH; AAV-CRE saline+AMPH vs. AAV-CRE baclo+AMPH; AAV-GFP saline+AMPH vs. AAV-CRE baclo+AMPH; AAV-GFP saline+AMPH vs. AAV-CRE saline+AMPH. Arrows indicate amphetamine/saline injection. **(d-f)** Loss of PRAF2-dependent behavioral effects in GB1-KO mice. The locomotor effect of AAV-PRAF2+GFP unilateral injection in the VTA of GB1-KO mice and control littermates (WT) was compared with that of AAV-CRE-GFP injection in the VTA of GB1<sup>fl/fl</sup> mice. Spontaneous locomotor activity of each mouse was measured two weeks before (Pre-test) or after (Test) unilateral injection of AAV-PRAF2+GFP or AAV-CRE-GFP in the VTA. In WT mice, PRAF2 induced a significant homogeneous enhancement of motor activity (video-tracking in **(d)**, quantification in **(e)** one-way ANOVA  $p=0,001$ ;  $n=10$ ). In GB1-KO mice Pre-test locomotor activity was enhanced on average, comparatively to WT animals, one-way ANOVA  $p=0,0365$  (with marked variations between individuals), but was not enhanced by exogenous PRAF2 expression in the VTA, one-way ANOVA  $p=0,371$ ;  $n=10$ . In GB1<sup>fl/fl</sup> mice CRE induced a significant enhancement of motor activity comparable to that caused by PRAF2 in wt mice, one-way ANOVA  $p=0,001$ . **(f)** Number and direction of turns recorded for WT and GB1-KO mice after

AAV-PRAF2+GFP injection in the VTA and for GB1<sup>fl/fl</sup> mice after AAV-CRE-GFP injection in the VTA. n.s: non significant.

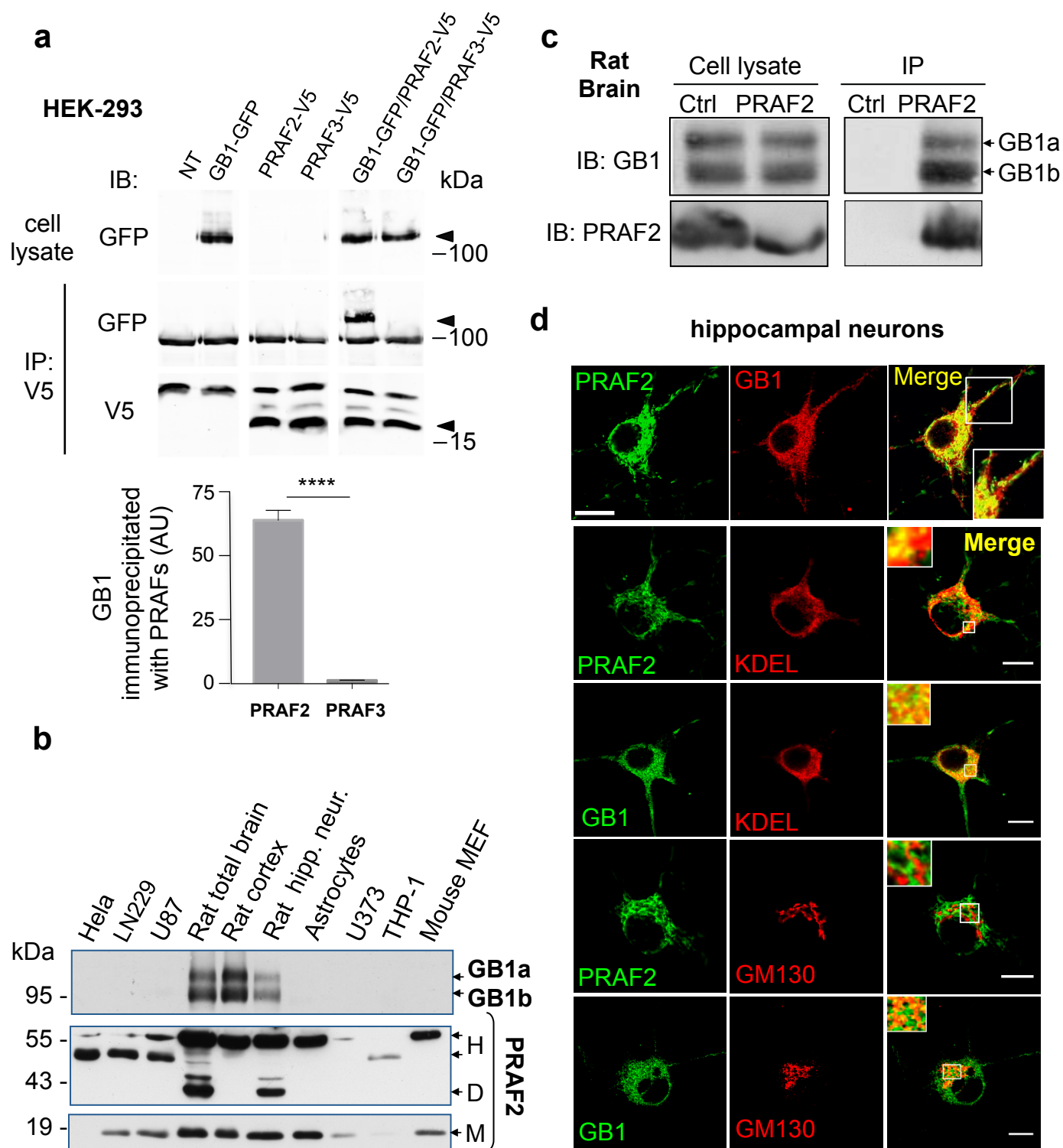


Figure 1

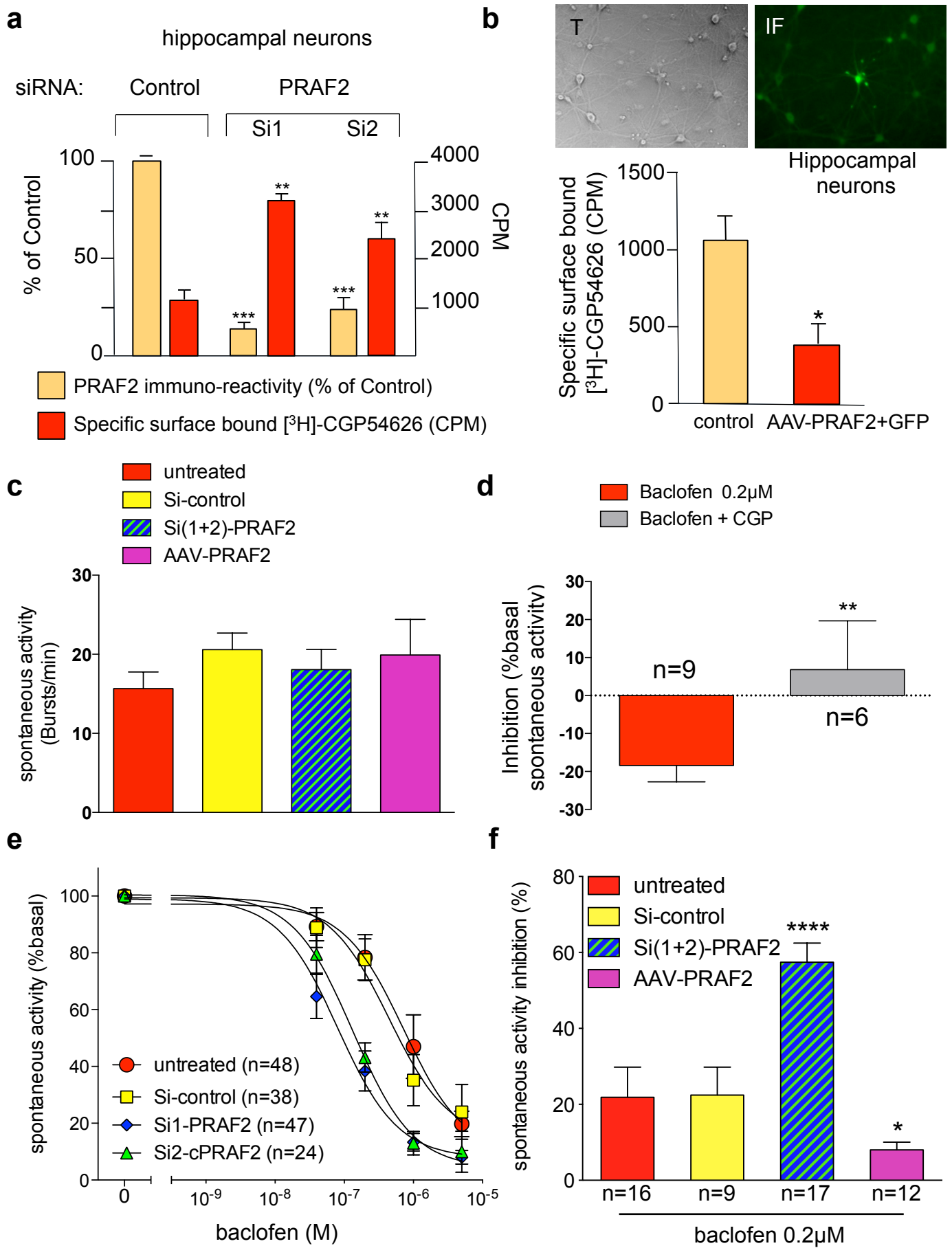


Figure 2

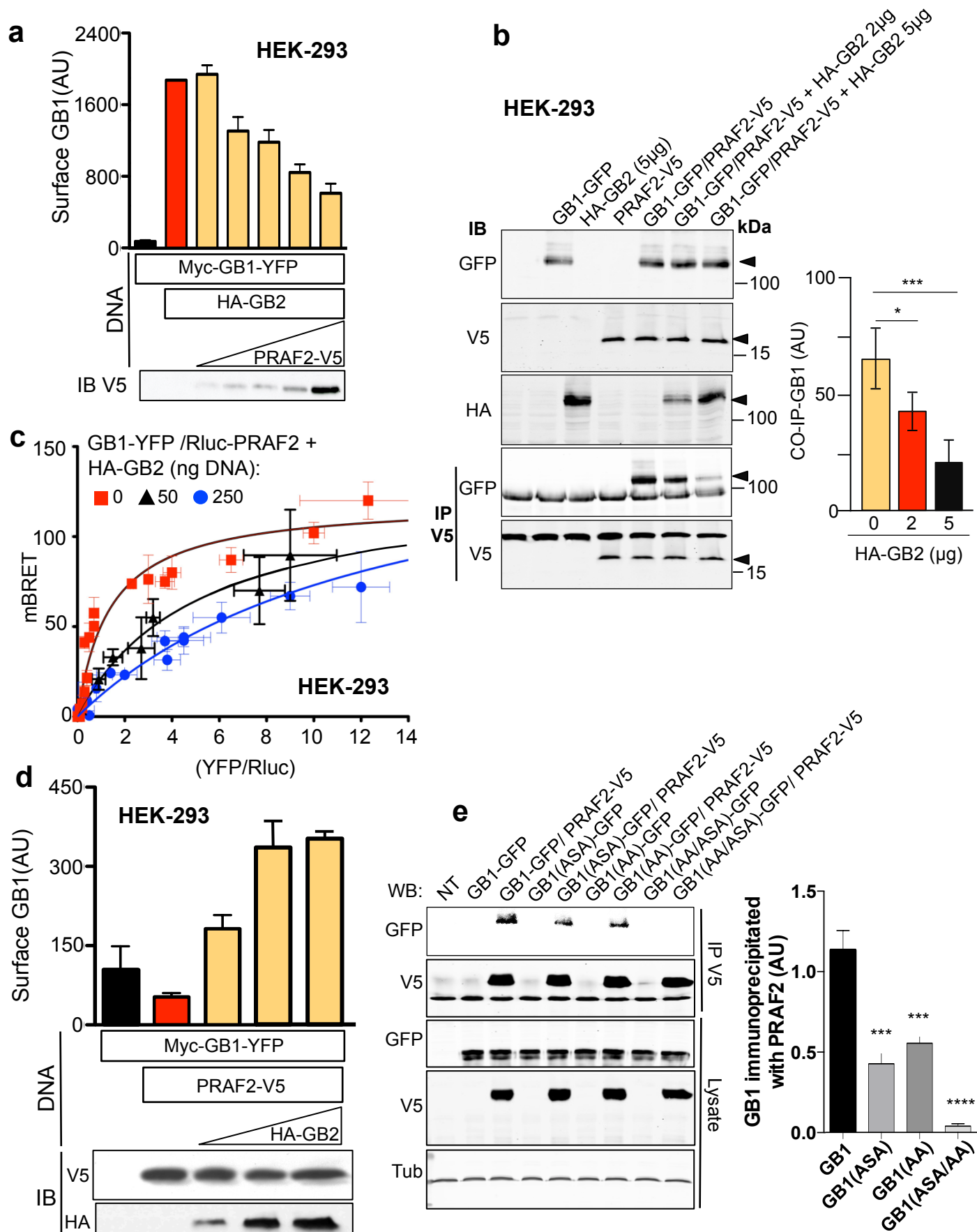
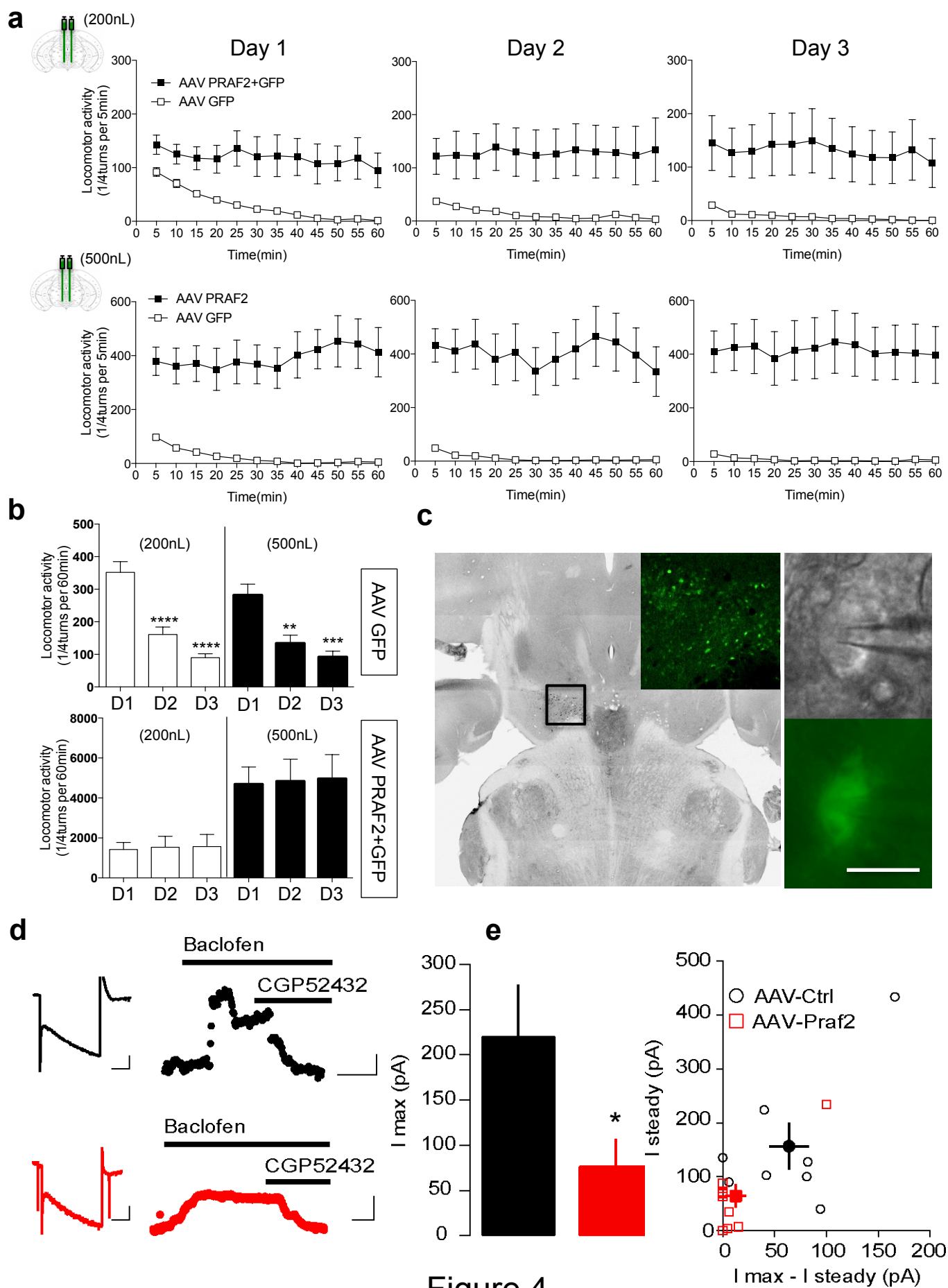


Figure 3





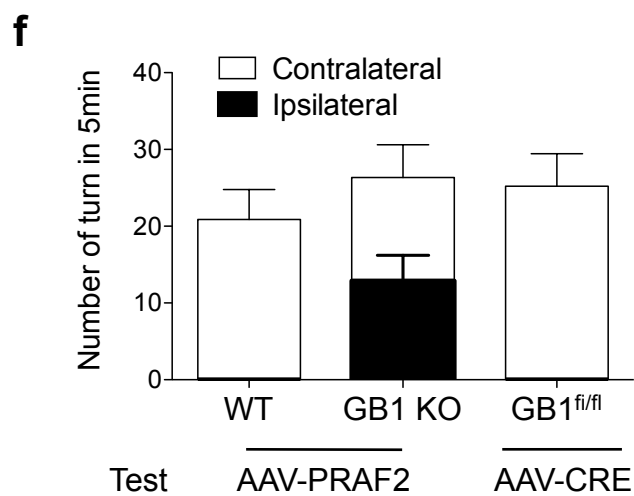
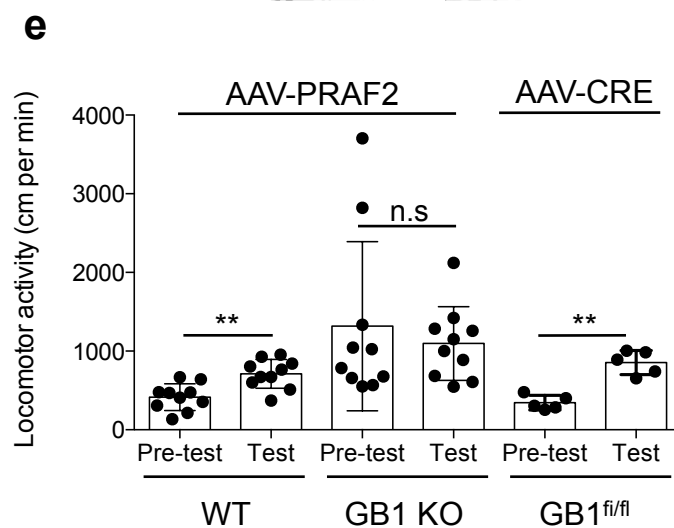
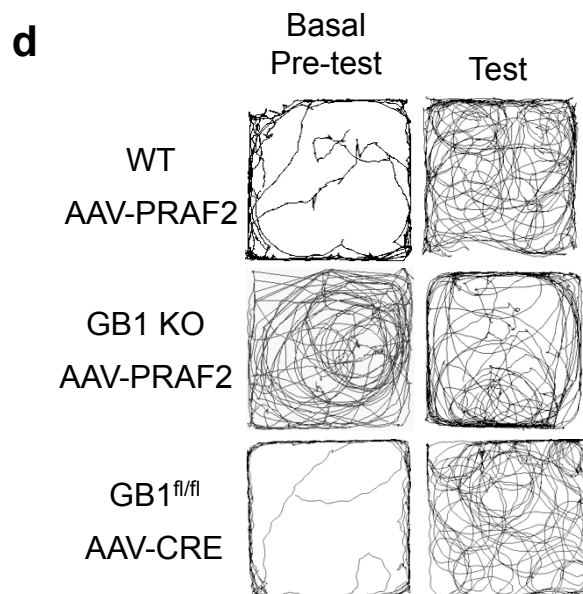
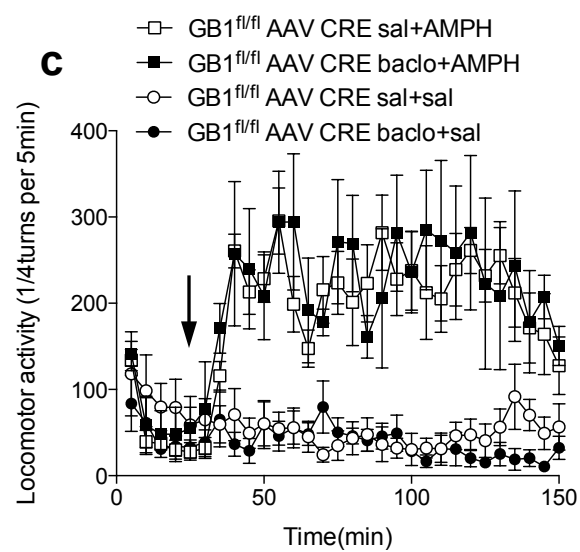
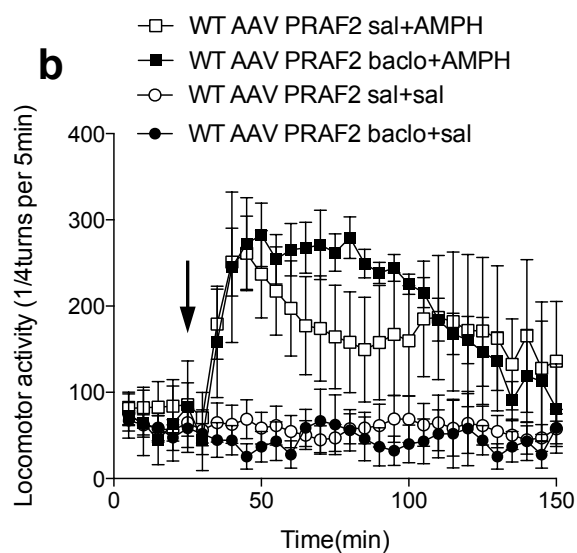
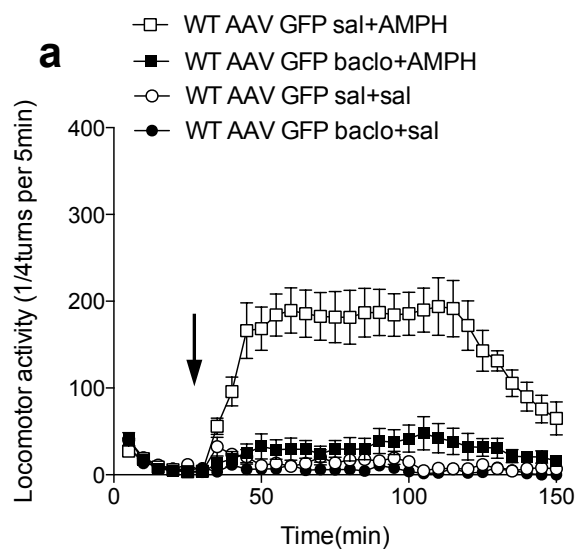


Figure 5